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Nicotinic acetylcholine receptors (rs1051730) gene polymorphism and surfactant protein D level in chronic obstructive pulmonary disease

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Chronic obstructive pulmonary disease (COPD) is one of the 10 main causes of morbidity and death in adult populations worldwide, the pathophysiology being airflow obstruction [1]. The integrity of the alveolar surface during the ventilation cycle is maintained by a number of secreted products that include surfactants, a conjugated blend of lipids (~90%) and proteins (~10%) that reduce surface tension. Four surfactant proteins (SPs): SP-A, SP-B, SP-C and SP-D are recognised, the latter (coded for by *SFTPD* at 10q22.3) also having immunological activity [2]. The WHO reports that smoking kills more than 5 million people every year, with >70% of deaths caused by lung cancer and COPD [3]. Despite this, only a small proportion of smokers develop symptomatic disease, suggesting that genetic and other factors might lead to increase susceptibility to COPD [4].

Nicotine functions via neuronal and peripheral nicotinic acetylcholine receptors (nAChRs). All nAChRs are ligand-gated ion channels composed of five transmembrane subunits formed from different combinations of 12 α (α2–α10) and β (β2–β4) subunits, each of which is encoded for by a single gene. *CHRNA3*, located at 15q25, codes for part of α subunit expressed in brain and peripheral tissues, and includes an SNP (rs1051730) that could change nAChRs function [5]. We hypothesised links between serum SP-D and nicotinic acetylcholine receptors (nAChRs) (rs1051730) (G/A) gene polymorphism in COPD.

We tested our hypothesis in 80 smokers with COPD and 80 age (mean [SD] 61.7 [6.0] vs. 60.8 [4.1], respectively, t-test $p = 0.25$) and gender-matched (64 men/16 women vs. 57 men/23 women, respectively, chi-squared $p = 0.19$) healthy non-smoker individuals. Exclusion criteria were acute exacerbation of COPD, respiratory failure and any other pulmonary diseases. Written consent was received from each enrolled subject, which was approved by the ethical committee of the Faculty of Medicine, Menoufia University. All subjects had full clinical and physical examinations including forced expiratory volume in one second % predicted (FEV1% predicted), forced vital capacity % predicted (FVC% predicted) and FEV1/FVC. Diagnosis of COPD was according to a clinical history suggestive of COPD and spirometry (the ratio of FEV1/FVC being less than 70%) and increase of post-bronchodilator FEV1 less than 12% [6]. A smoking index was calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked [7]. Serum SP-D was measured by ELISA (R&D Systems, Minneapolis, MN, USA). Genotyping of nAChRs (rs1051730) (G/A) used TaqMan allelic discrimination assay (real-time PCR). The qPCR Master Mix (40X), probes and primers were provided from Thermo Fisher Scientific (Foster City, CA, USA). Five millilitres of venous blood was obtained from all subjects and distributed as follows: 3 ml in a plain tube, from which serum was separated and stored at −80°C until measuring of SP-D level. Two millilitres was transferred into an EDTA tube and stored at −20°C until the extraction of DNA and further molecular analysis. DNA was eluted from whole blood utilizing Zymo Research Quick-g DNA, Genomic DNA purification kit (Irvine, CA, USA). The quality of the isolated DNA was evaluated utilizing a nano-drop. The nAChRs TaqMan probe sequence was AGCAGTTGTACTTGATGTCGTGTTT[A/G]TAGCCTGGGGC TTTGATGATGGCCC. The sequences of the forward and reverse primers were 5′AGGGAGAGGAGGACAGAAA-3′ and 5′AAGGACTATTGGGAGAGCG-3′, respectively. Ten microlitres of master mix was added to 1.25 µl of the genotyping assay and 3.75 µl of DNAase-free water. 5 µl of genomic DNA extract for every sample and 5 µl of DNAase-free water for the negative control were applied. The following conditions were used: Initial denaturation was carried out at 95°C for 10 minutes accompanied by 40 cycles of denaturation at 94°C for 15 seconds, primer annealing at 50°C for 60 seconds followed by extension at 72°C for 2 minutes and the final extension at 72°C for 1 minute. Analysis of data was completed using 7500 Real-Time PCR, version 2.0.1 (Applied Biosystems, Foster

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City, CA, USA). The data were tabulated and analysed by SPSS version 16 using Chi-square, Student's t and Mann–Whitney tests. Significance was assessed at P value ≤0.05.

Table 1 shows significant statistical differences regarding the spirometry (as expected [8]), BMI and SP-D in COPD patients compared to controls. The smoking index in the patients was 21 (10–35) pack years. The AG and AA genotypes in nAchR rs1051730 were both strongly linked to COPD with odds ratios (95% CIs) of 4.87 (2.07–11.45) and 6.15 (2.76–13.72), respectively (both p <0.001). The A allele was also strongly linked to COPD with an odd's ratio (95% CI) of 3.65 (2.29–5.78) (p <0.001). Table 2 shows that sex, BMI, FVC% and SP-D are strongly linked to nAChRs (rs1051730) genotypes. Table 3 shows factors most likely to predict COPD: the strongest were (in order) nAChRs (rs1051730) AA genotype, AG genotype, FEV1% and SP-D.

We hypothesised links between serum SP-D and nicotinic acetylcholine receptors (nAChRs) (rs1051730) (G/A) gene polymorphism in COPD, finding both to be strongly linked. Levels of serum SP-D were around 75% higher in COPD, and the A nucleotide highly expressed – more than double for the AA genotype and almost double for the A allele. In the analysis of patients according to genotype, there were marked trend links with sex, BMI and SP-D, suggesting a mechanism. This may be so for BMI and SP-D, but it is unclear why there is a strong effect of sex (i.e. no women with the AA genotype, 80% of those with the GG genotype being women) unless there is an unknown linkage with X or Y chromosomes. However, the effect of BMI disappeared on multivariate analysis, which found that AG and AA genotypes were the strongest predictors of COPD.

Our data support and extend those of others reporting that SP-D is capable to distinguish COPD patients from those consulting for other pulmonary disorders and from controls, especially among smokers [9]. Serum SP-D levels are increased in smokers with COPD compared to non-smokers and correlate positively with current smoking status. SP-D expression is correlated with pulmonary function and is increased in stable COPD, with higher levels observed in acute exacerbation. Change in SP-D level is associated with an improvement of COPD symptoms; this suggests that serum SP-D might be utilized as a lung-specific biomarker in COPD progression [10]. As regards the nAChRs (rs1051730) (G/A) SNP, there was a significant proportional increase of the AA genotype and A allele in COPD compared to the controls. The results also showed that both AA and AG genotypes elevate the likelihood of COPD with a significant decrease of FEV1% in these genotypes in contrast to GG genotype. This may be explained by the expansion of airway obstruction and excess mucus due to change of receptor function. This is in agreeing with Perez-Morales et al. [11] who reported links between another nAChR, nicotine dependence and COPD.

Recent evidence proposed that stimulation of nAChRs results in complete inhibition of the ion channel function of ATP receptor, so the surfactant restrains inflammation and maturation of IL-1β production through a mechanism including nAChRs [12]. Thorgeirsson et al. [13] demonstrated significant correlation with lower BMI and rs1051730 in smokers. The effect of BMI is probably through the effect of the polymorphism on smoking behaviour and the increase in metabolic rate and appetite suppression attributable to nicotine, which may explain our results of association of AA genotype with the lowest BMI. Although limited by small numbers, our data show strong effects, and these must be confirmed, especially the differences in the sexes according to genotype, and we cannot exclude the possibility that some of the variances in SP-D may be due to sex, BMI and smoking, and not to the genotypes. We also acknowledge the weakness of the lack of a group of COPD non-smokers, and that we cannot analyse smoking by sex due to small numbers. Nevertheless, the work represents an advance in biomedical science because it shows that AA genotype of nAChRs (rs1051730) and elevated serum level of SP-D predict the presence of COPD and so may be biomarkers.

Table 1. BMI, Spirometry, surfactant D and nAChRs analysis in cases and controls.

Variables	COPD group N = 80	Control group N = 80
BMI (Kg/m^2)	24.1 [4.0]	29.9[2.4]
FEV1%	46 [10]	97 [7]
FVC%	66[6]	87[4]
FEV1/FVC	59[6]	85 [3]
SP-D (ng/ml)	87.2[11.9]	49.7[12.3]
nAchR genotype (n,%)		
GG	15 (18.8%)	45 (45.2)
AG	26 (32.5%)	16 (20.0)
AA	39 (48.8%)	19 (23.8)
nAchR allele (n,%)		
G	56 (35.0)	106 (66.3)
A	104 (65.0)	54 (33.7)

All differences: p < 0.001

Table 2. Analysis of BMI, sex, spirometry and surfactant D according to nAChRs (rs1051730) genotype.

	nAChRs (rs1051730) genotype			
Variables	AA (n = 39)	AG (n = 26)	GG (n = 15)	P value
Men/women (n)	39/0	22/4	3/12	< 0.001
BMI (Kg/m^2)	22.6 [2.6]	24.3 [4.5]	27.8 [4.0]	< 0.001
FEV1%	47[12]	42[8]	50[8]	0.05
FVC%	67[6]	63[5]	68[7]	0.005
FEV1/FVC	61[5]	57[6]	59[7]	0.08
SP-D (ng/ml)	97.1 [5.3]	81.7 [6.5]	71.0 [6.1]	< 0.001

Table 3. Binary logistic regression for independent risk factors for the occurrence of COPD.

Variable	OR (95% CI)	P value
BMI (Kg/m^2)	0.95 (0.51–2.31)	0.35
FEV1%	2.65 (1.52–16.14)	0.01
FVC%	1.64 (0.69–5.74)	0.04
FEV1/FVC	1.85 (1.40–9.88)	0.03
SP-D (ng/ml)	2.02 (1.03–5.47)	0.01
nAChRs genotypes		
AG	3.27 (1.87–9.51)	<0.001
AA	5.06 (2.61–11.73	<0.001

Disclosure statement

No potential conflict of interest was reported by the authors.

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